ANNEX 5

DRAFT
THE PREPARATION OF TEST MEDIA WITH POORLY SOLUBLE COMPLEX MIXTURES FOR MARINE TOXICITY TESTS

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1 INTRODUCTION

This standard operating procedure was originally designed for use in the preparation of test media for hydrocarbon mixtures. however, it is suitable for the preparation of water accommodated fractions (WAF) of other poorly soluble complex mixtures in seawater. Generally, the method follows the recommendations for testing difficult substances according to EU regulations provided by Whitehouse & Mallet (1993). It is based on methods developed by Girling (1989) and Girling et al. (1994) and has been accepted by Concawe (1993) as a standard method for oil products. This procedure has been in use at TNO for at least 15 years.

The technique involves rapid stirring of test substances in test media, and can be considered as a high energy method of preparing WAF's or aqueous extracts.

2 TERMINOLOGY AND DEFINITIONS

- The term **test substance** is used here to describe mixtures, whether simple or complex and includes both natural mixtures, such as oils and isomeric mixtures from a chemical process, as well as artificial or deliberate mixtures such as preparations.

- The term **water accommodated fraction** (WAF) refers exclusively to mixtures and is not applicable to pure substances (equivalent term: aqueous extracts).

- Although it contains dissolved material, a WAF can best be referred to in reporting as the **test medium** and not as the "test solution".

- The initial concentrations mixed in seawater should be consistently referred to as **loading rates** when presenting results and not as "test concentrations", as the initial amount was never present in the media actually tested.

3 PRINCIPLE

3.1 The test substance is first homogenised thoroughly, bearing in mind that substances with a tendency to emulsify may have to be rolled or shaken for several hours and then weighed out immediately.

3.2 As a water accommodated fraction comprises a differential equilibrium of components, between the undissolved and the dissolved phases, each test concentration/loading rate of a series must be prepared separately. Dilution of a single stock is not acceptable.

3.3 Accurately weighed amounts of homogeneous test substance are thoroughly mixed with a given volume of seawater using a magnetic stirrer, i.e., long enough to obtain a partial equilibrium

between the seawater and the test substance. The mixture is then left to stand for a further period, to allow for phase separation. Material will float, settle to the bottom or remain in suspension, depending on its specific gravity. If in doubt the equilibrium may be determined by chemical analysis of relevant components.

3.4 Following phase separation the required volume of test medium is tapped off from the middle of the mixing vessel. This fraction is called the "water accommodated fraction" (WAF) in conformity with Ref 1. The WAF may contain very small (invisible) droplets or particles.

3.5 The WAF is used directly for testing except in cases where it is judged to be sufficiently turbid as to cause physical hampering of the test organisms (particularly crustaceans). In such cases, it may be filtered through a glass wool plug. In order to prevent losses of sparingly soluble compounds by evaporation (filtration under low pressure) or adsorption (in filter material), the WAF **may not be** filtered through a fine membrane or other filter. Centrifugation may be considered if no other alternatives are available.

3.6 Substances containing volatile components may have to be mixed and tested in sealed vessels. Substances which degrade rapidly, may need shorter phase separation times.

4 APPARATUS

Ordinary laboratory apparatus is used, in particular:

- magnetic stirring apparatus
- glass stoppered Erlenmeyer flasks with a glass tap assembly ca. 3cm above the base
- laboratory balance
- glass microscope cover slips
- time clock(s) for electrical power (if possible)

5 PREPARATION OF TEST MEDIA

Remember to start the preparation of the media one day (20h + 4h) in advance of the test start date.

5.1 Homogenise the test substance thoroughly, e.g., by rolling overnight on a roller bank in a cool environment (15±2°C).

5.2 Accurately weigh the necessary amounts of test substance. Small amounts may be weighed on a glass microscope cover slip (one amount for each test solution to be prepared); avoid the use of non-inert materials to transfer the test substance.

5.3 Fill Erlenmeyer flasks (with a glass stopper) almost completely with a known amount of seawater (the seawater type and temperature of choice for the test). Introduce a suitable teflon/glass magnetic stirring rod and place each of these flasks on a magnetic stirrer at about the test temperature, making sure that the vortex reaches a depth of 1/3 of the water column. The depth of the vortex is important in ensuring that the individual loading rates are stirred with approximately equal energy.

5.4 Introduce the weighed amounts of test substance, one for each flask, when the seawater is already stirring; this may improve the mixing procedure.

5.5 The preparation of the WAFs is generally carried out in the dark as some substances, e.g., hydrocarbons, may be sensitive to photo-oxidation.

5.6 Stir for 16-20 h, followed by 4 h standing for phase separation. If possible, stir at some degrees below the test temperature, as stirring may slightly warm the seawater.

5.7 Following the period allowed for phase separation tap the WAFs from the middle of the water column directly into the test vessels (not more than 70% of the volume). Filtration should be avoided at all costs.

5.8 This procedure is followed on each occasion that the test media are replaced; for a 96 h (fish) test with daily renewal, the test media are prepared 4 times.

6 REPORTING

Refer accurately to the procedure in the report:

- state that water accommodated fractions were used;
- give the stirring and standing times;
- quote the results as lethal loading rates and effect loading rates (LL50, EL50, NOEL) etc., not as LC/EC50s or NOECs.

7 REFERENCES

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Ecotoxicology testing of petroleum products; test methodology.
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Aquatic toxicity testing for notification of new substances: an advisory document dealing with difficult substances. Report to the chemical notification unit, Department of the Environment (UK).
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